



ELSEVIER

International Journal of
Food Microbiology 30 (1996) 217–229

International Journal
of Food Microbiology

Enterotoxigenic *Escherichia coli* detected in foods by PCR and an enzyme-linked oligonucleotide probe¹

M.Y. Deng^{a,2}, D.O. Cliver^{b,3}, S.P. Day^c, P.M. Fratamico^{a,*}

^aEastern Regional Research Center, USDA, Agricultural Research Service, Wyndmoor, PA 19038, USA

^bFood Research Institute, University of Wisconsin-Madison, Madison, WI 53706, USA

^cViroMed Laboratories Inc., Minneapolis, MN 55343, USA

Received 14 February 1995; revised 17 July 1995; accepted 2 August 1995

Abstract

A polymerase chain reaction (PCR) and an enzyme-linked oligonucleotide probe hybridization assay were developed for the detection of enterotoxigenic *Escherichia coli* (ETEC) in ground beef, chicken, pork and raw milk. Two synthetic primers, one of which was biotinylated, were used in the PCR to amplify a fragment of the *E. coli* heat-labile enterotoxin (LT) gene. The identity of the amplified products was confirmed by liquid hybridization using a horseradish peroxidase-linked internal oligonucleotide probe in a 96-well microplate coated with streptavidin. The final quantitation of the PCR products was performed by a colorimetric reaction. Under established conditions (including 1 min at 60°C for primer annealing and extension in PCR cycles), this method detected all 7 LT-producing *E. coli* pathogenic for humans, but did not detect all 7 LT-positive *E. coli* of animal origin, 3 *E. coli* strains that do not produce LT, and 9 other bacteria. Under less stringent PCR conditions (55°C for annealing and extension), 2 strains of LT-producing *E. coli* of porcine origin were detected while the results of other bacterial strains remained unchanged. In pure

* Corresponding author, Microbial Food Safety Research Unit, Eastern Regional Research Center, USDA Agricultural Research Service, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA. Tel: +1 215 2336525; fax: +1 215 2336581.

¹ Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

² Present address: IDEXX Laboratories Inc., 1 IDEXX Drive, Westbrook, ME 04092, USA.

³ Present address: Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.

cultures, the detection limit of the method was 1.4 colony forming units (CFU). Prior to PCR amplification, all food samples inoculated with an LT-producing ETEC, were subjected to enrichment in brain heart infusion broth for 8 h at 37°C. From these cultures, 10 µl was heated at 95°C for 10 min and directly used in the PCR. An initial inoculum of as few as 1.2 to 12 CFU of the LT-producing ETEC per 25 g (or ml) of food sample gave a positive reaction.

Keywords: Enterotoxigenic *Escherichia coli*; Heat-labile enterotoxin gene; Polymerase chain reaction; Enzyme-linked oligonucleotide probe hybridization; Food samples

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are a frequent cause of diarrheal disease throughout the world (Neill et al., 1994). These strains cause diarrhea by producing a heat-labile enterotoxin (LT), a heat-stable enterotoxin (ST), or both (Evans et al., 1975; Sack, 1975). Illness caused by ETEC has been associated with the ingestion of contaminated food and water (Levine, 1987). Immunological assays and tissue culture techniques currently used for detection of ETEC (Sack and Sack, 1975; Yolken et al., 1977) are non-specific and insensitive, and thus not used routinely (Victor et al., 1991). Cloned DNA probes and oligonucleotides have been used in colony hybridization for LT and ST enterotoxins; but these methods, although specific, require the use of radiolabeled probes (Candrian et al., 1991; Jablonski et al., 1986). In contrast, the polymerase chain reaction (PCR) allows a million-fold primer-directed enzymatic in-vitro amplification of specific DNA sequences within hours (Saiki et al., 1988). Although the majority of reports describing the use of PCR for the detection of pathogens have been in the area of clinical diagnostics, there have been some results concerning the application of PCR in food microbiology (Gannon et al., 1992; Koch et al., 1993; Lampel et al., 1990; Mahon et al., 1994; Wang et al., 1992), including detection of ETEC in minced meat (Wernars et al., 1991). However, PCR primers used in the above-mentioned study on ETEC were based on the LT gene of porcine origin (Wernars et al., 1991), which shows sequence divergence (Leong et al., 1985). We describe here a method that uses PCR and an enzyme-linked oligonucleotide probe hybridization (ELOPH) assay for the detection of LT-producing ETEC in ground beef, chicken, pork and raw milk. PCR primers designed are specific for the LT gene of *E. coli* that are pathogenic for humans. The protocol consists of a short-term (8 h) enrichment culturing of food samples, high-temperature denaturation of the cultures, and direct PCR amplification without DNA extraction.

2. Materials and methods

2.1. Bacterial strains

A complete list of bacterial strains and their sources appears in Table 1. All bacterial strains were grown in Luria-Bertani (LB) medium (Difco Laboratories, Detroit, MI) overnight at 37°C. *E. coli*, strain 43886, an LT-producing ETEC,

which was used throughout this study, was enumerated using the pour plate technique described by Swanson et al. (1992).

2.2. Synthetic oligonucleotides

The sequence information of the *toxB* region (375 bp) of the LT gene, which encodes the B subunit of LT produced by ETEC pathogenic for humans was from Yamamoto and Yokota (1983) and Olive (1989). In the early stages of this study, PCR amplification primers LT1 (nucleotides 70–89, 5'-CAGTCTATTACAGAACTATG) and LT2 (nucleotides 347–366, 5'-CCATACTGATTGCCGCAATT) were purchased from Genemed Biotechnologies Inc. (San Francisco, CA). Later, the primers were synthesized by the University of Wisconsin Biotechnology Center, Madison, WI. The reverse primer (LT2) was labeled with biotin at its 5' end. The primer pair amplifies a 298-bp fragment in the *toxB* region of the LT gene. A horseradish peroxidase (HRP)-linked internal oligonucleotide probe (nucleotides 193–214, 5'-AGCGGCG-CAACATTTTCAGGTCG) was synthesized by Synthetic Genetics (San Diego, CA).

2.3. PCR conditions

A 10- μ l portion of a bacterial culture in LB medium or enrichment culture of a food sample was added to a 0.5-ml PCR tube and heated in a Model 480 Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) to 95°C for 10 min to lyse the bacteria, denature the DNA, and inactivate PCR inhibitors possibly present in the sample. Ninety μ l of a reaction mixture containing 1 \times PCR Buffer II (Perkin-Elmer Cetus), 2.0 mM MgCl₂, each of the four deoxynucleoside triphosphates (dNTPs) at a concentration of 0.2 mM, 0.5 μ M of each primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) were added to the tube. The reaction mixtures were overlaid with sterile mineral oil and then subjected to 35 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min, followed by an additional 7-min extension at 60°C. A negative control (sterile growth medium) was included in each experiment.

2.4. Analysis of PCR products

The PCR products were analyzed by electrophoresis on 2% agarose gels with ethidium bromide staining (Maniatis et al., 1982). To confirm the identity of the PCR products, the amplified material was subjected to an ELOPH assay. The procedure used was a modification of one described by Holodniy et al. (1992) for determining human immunodeficiency virus *gag* DNA copy number. Wells of Reacti-Bind™ streptavidin coated 96-well polystyrene strip plates (Pierce, Rockford, IL) were filled with 300 μ l of a blocking solution containing 5 \times Denhardt's solution, 1% gelatin (Sigma Chemical Co., St. Louis, MO), and 250 μ g/ml sheared herring sperm DNA (Promega, Madison, WI) and the plates were incubated overnight at 4°C. These plates could then be stored for several weeks at 4°C. Immediately before use, the blocking solution was aspirated from each well, and 65 μ l of a hybridization solution containing 5 \times saline sodium phosphate EDTA, 5 \times Denhardt's solution and 1 pmol of the HRP-linked LT-specific internal probe was added to each well.

After the PCR products were heated at 95°C for 5 min and cooled rapidly on ice, a 10- μ l portion of each product was transferred to a well of the 96-well microplate. The plate was then incubated at 42°C for 1 h to allow PCR product binding and probe hybridization. The wells of the microplate were washed 20 times with phosphate buffered saline, pH 7.4, containing 0.05% Tween-20 using a Dynatech Miniwash (Dynatech Laboratories Inc., Alexandria, VA). One hundred and fifty μ l of the HRP substrate, *o*-phenylene-diamine (OPD, Sigma), prepared with 0.6 mg/ml in 0.1 M citrate buffer (pH 5.5) containing 0.03% hydrogen peroxide, was added to each well. After 10 min, the reaction was stopped with 100 μ l of 1 N H₂SO₄ and the optical density (OD) at 490 nm of each well was measured with a microplate reader (Molecular Devices Corp., Menlo Park, CA). Unreacted substrate was used as a reference. A positive/negative ratio was determined as follows: $P/N = OD \text{ sample PCR} / OD \text{ negative control}$, where the OD sample PCR and the OD negative control are the OD at 490 nm of the PCR products of the sample tested and of the negative control, respectively. All assays were performed in duplicate, and the results were averaged. The ELOPH assay of a sample was regarded as positive if the P/N ratio was 2.0 or higher.

When the PCR products of a sample were the expected length of 298 bp as determined by ethidium bromide staining of the gel and showed a positive result in the ELOPH assay, the sample was considered positive for LT-producing ETEC.

2.5. Detection of LT-producing *E. coli* in foods

Ground beef, chicken, pork and raw milk were obtained from a local grocery store and tested by PCR for indigenous LT-producing *E. coli* before being used. No indigenous LT-producing *E. coli* were detected in these foods. An overnight culture of LT-producing *E. coli* (strain 43886) in LB medium, at a concentration of 4.7×10^8 colony forming units (CFU)/ml, was used in this experiment. Ten 25-g samples each of ground beef, chicken and pork were inoculated with 2.5 ml of 10-fold dilutions of *E. coli* prepared in Butterfield's phosphate diluent (BPD), pH 7.2, using a 5-ml plastic syringe with an 18-gauge (1.2 mm) hypodermic needle. Ten 25-ml samples of raw milk were each inoculated with 2.5 ml of the same dilutions as for the ground beef, chicken and pork samples. One uninoculated 25-g (or ml) sample of each type of food was used as a control. Each sample received 222.5 ml of brain heart infusion (BHI) medium and was processed with a Stomacher (Model BA601, Cooke Laboratory Products, Alexandria, VA) for 60 s. All samples were incubated for 8 h at 37°C on a rotary shaker at 100 rev./min. Ten μ l of culture from each sample was heated and subjected to PCR as described above.

3. Results

3.1. Specificity of the PCR

Under the PCR conditions described in Section 2, the primer pair generated PCR products of the expected size of 298 bp with all 7 LT-producing ETEC pathogenic

for humans, but not with all 7 LT-positive *E. coli* of animal origin (4 porcine isolates and 1 isolate from each of alpaca, bovine and ostrich), 3 *E. coli* strains that do not produce LT and 9 other bacteria (Table 1). However, when the annealing and extension temperature in the PCR thermocycling protocol was lowered from 60 to 55°C, 2 strains of the 4 porcine isolates of LT-producing *E. coli* (serotype O149:H10, strain 87.0197, and serotype O149:NM, strain 86.0804) were detectable while results with all other bacterial strains remained unchanged. The amplified products obtained with the LT-producing ETEC strains were confirmed to include the appropriate LT gene sequence by a positive result in the ELOPH assay. A typical agarose gel analysis of PCR products obtained from some *E. coli* cultures is shown in Fig. 1A.

3.2. Sensitivity of the PCR

To evaluate the sensitivity of the PCR, serial half-logarithmic dilutions prepared in BPD, pH 7.2, of an overnight culture of the LT-producing *E. coli* (strain 43886) of known concentration in CFU/ml were tested. As shown in Fig. 1B, with serial dilutions of the bacterial culture down to 1.4 CFU, the PCR gave a positive signal in an ethidium bromide-stained agarose gel (lane j). Samples containing less than 1.4 CFU (Fig. 1B, lanes k and l) and the negative control (Fig. 1B, lane N) did not display a DNA band.

When the PCR products of all samples obtained in this experiment were analyzed by the ELOPH assay, all samples containing ≥ 1.4 CFU showed a P/N ratio of 2.0 or higher. There was a positive correlation between the number of CFU of the LT-producing *E. coli* and the OD of the PCR products (Fig. 2). The correlation coefficient was +0.593. Visualization of amplification products by ethidium bromide staining of agarose gels and quantitation of the PCR products by the colorimetric enzymatic reaction gave approximately the same sensitivity of 1.4 CFU. Similar results were obtained when this experiment was repeated.

3.3. Detection of LT-producing *E. coli* in foods

Results of testing experimentally inoculated and control food samples for the presence of LT-producing ETEC are shown in Table 2. For inoculated ground beef and chicken samples, with serial dilutions of the initial inoculum down to 12 CFU per 25-g sample, the PCR gave a positive reaction. For the inoculated pork and raw milk samples, an initial inoculum of as few as 1.2 CFU per 25-g (or ml) displayed a positive signal. No signals were obtained with all control samples that were not inoculated with ETEC. These PCR results were confirmed by the ELOPH assay.

4. Discussion

We describe here a method for the detection of LT-producing *E. coli* in ground beef, chicken, pork and raw milk samples using PCR and an ELOPH assay. It

consists of a short-term (8 h) enrichment culturing of a food sample, high-temperature denaturation of the cultures, and PCR amplification of a 298-bp DNA fragment of the *tox*B region of the LT gene. The PCR primer pair used in this study

Table 1

Bacterial strains used in this study and PCR results^a

Bacterium	Strain	Source	PCR results
<i>E. coli</i> O2:NM, LT ⁺ ^b	91.1398	ECRC ^c (human)	+ ^d
<i>E. coli</i> O6:H ⁻ , LT ⁺	87.0446	ECRC (human)	+
<i>E. coli</i> O7:NM, LT ⁺	85.1934	ECRC (human)	+
<i>E. coli</i> O25:NM, LT ⁺	43886	ATCC ^e (human)	+
<i>E. coli</i> O25:NM, LT ⁺	91.0988	ECRC (human)	+
<i>E. coli</i> O159:H ⁻ , LT ⁺	84.0399	ECRC (human)	+
<i>E. coli</i> O78:H11, LT ⁺ ,ST ⁺ ^f	35401	ATCC (human)	+
<i>E. coli</i> O78:H12, ST ⁺ ^g	43896	ATCC (human)	— ^h
<i>E. coli</i> O149:H10, LT ⁺	87.0197	ECRC (porcine)	— ⁱ
<i>E. coli</i> O149:NM, LT ⁺	86.0804	ECRC (porcine)	— ^j
<i>E. coli</i> O107:H11, LT ⁺	92.0216	ECRC (porcine)	—
<i>E. coli</i> O138:NM, LT ⁺	93.0014	ECRC (porcine)	—
<i>E. coli</i> O153:H ⁻ , LT ⁺	89.0802	ECRC (alpaca)	—
<i>E. coli</i> (not serotyped), LT ⁺	91.0705	ECRC (bovine)	—
<i>E. coli</i> O45:H ⁻ , LT ⁺	91.1775	ECRC (ostrich)	—
<i>E. coli</i> O157:H7	43895	ATCC (meat)	—
<i>E. coli</i> non-pathogenic	FRIk1	FRI ^j (unknown)	—
<i>Salmonella typhimurium</i>	FRIk49	FRI (human)	—
<i>Yersinia enterocolitica</i>	FRIk34	FRI (unknown)	—
<i>Vibrio vulnificus</i>	FRIk126	FRI (human)	—
<i>Shigella sonnei</i>	FRIfv02	FRI (human)	—
<i>Listeria monocytogenes</i>	FRIfv04	FRI (food)	—
<i>Bacillus cereus</i>	FRIfv13	FRI (environment)	—
<i>Bacillus subtilis</i>	FRIfv18	FRI (environment)	—
<i>Enterococcus faecium</i>	FRIfv25	FRI (environment)	—
<i>Pseudomonas alcaligenes</i>	FRIfv26	FRI (environment)	—

^aThe thermocycling protocol for the PCR consisted of 35 cycles of denaturation at 95°C for 1 min and annealing and extension at 60°C for 1 min, followed by an additional 7-min extension at 60°C.

^bProduces the heat-labile enterotoxin (LT) (determined by the provider of the bacterium).

^c*E. coli* Reference Center, the Pennsylvania State University, University Park, PA.

^d+, positive. When the PCR products of a sample were the expected length of 298 bp as determined by ethidium bromide staining of the agarose gel and showed a positive result in the ELOPH assay performed as described in Section 2, the samples were considered positive for LT-producing *E. coli*.

^eATCC, the American Type Culture Collection, Rockville, MD.

^fProduces both LT and the heat-stable enterotoxin (ST) (determined by ATCC).

^gProduces only ST (determined by ATCC).

^h—, negative. If a sample produced neither a DNA band at the expected position (298 bp) nor a positive result in the ELOPH assay, the sample was considered negative for LT-producing *E. coli*.

ⁱA positive result was obtained when the annealing and extension temperature was lowered to 55°C, with other conditions in the thermocycling protocol remaining the same.

^jFRI, Food Research Institute, Madison, WI.

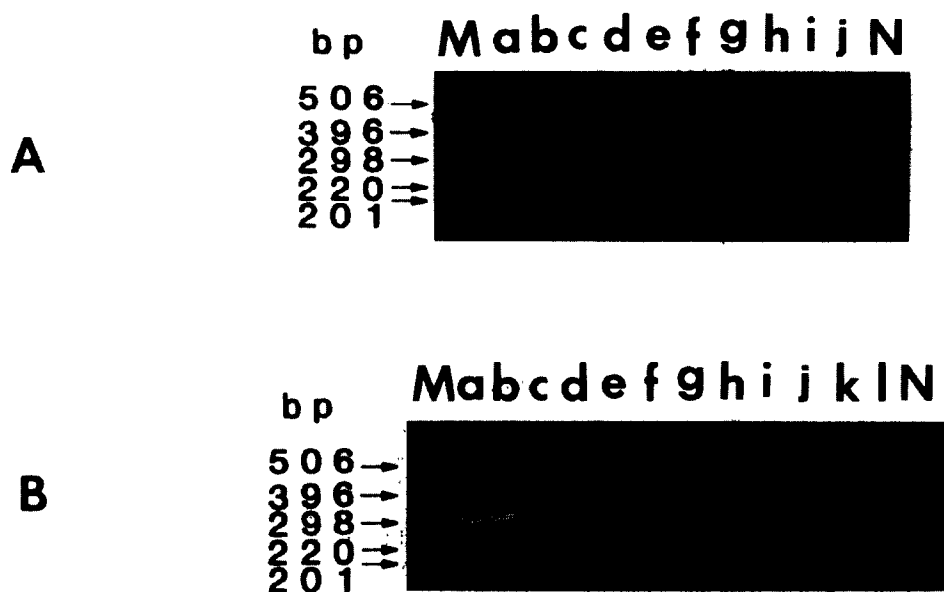


Fig. 1. Ethidium bromide-stained agarose gel showing PCR products obtained from different *E. coli* cultures (A) and from serial half-logarithmic dilutions of the heat-labile enterotoxin (LT)-producing *E. coli*, serotype O25:NM, strain 43886 (B). All bacterial strains were grown in Luria-Bertani (LB) medium (Difco) overnight at 37°C before being tested by PCR using the procedure described in Section 2. Lanes in A: M, DNA size marker (1 kb ladder, GIBCO, BRL); a, serotype O2:NM, LT⁺, strain 91.1398; b, serotype O6:H⁺, LT⁺, strain 87.0446; c, serotype O7:NM, LT⁺, strain 85.1394; d, serotype O25:NM, LT⁺, strain 43886; e, serotype O25:NM, LT⁺, strain 91.0988; f, serotype O159:H⁺, LT⁺, strain 84.0399; g, serotype O78:H11, LT⁺ and ST⁺, strain 35401; h, serotype O78:H12, ST⁺, strain 43896; i, serotype O157:H7, strain 43895; j, non-pathogenic *E. coli*, strain FRLk1; and N, negative control (LB medium). Lanes in B: M, DNA size marker; a, PCR products obtained from 4.4×10^4 CFU of the tested organism; b, 1.4×10^4 CFU; c, 4.4×10^3 CFU; d, 1.4×10^3 CFU; e, 4.4×10^2 CFU; f, 1.4×10^2 CFU; g, 44 CFU; h, 14 CFU; i, 4.4 CFU; j, 1.4 CFU; k, 0.44 CFU; l, 0.14 CFU; and N, negative control (Butterfield's phosphate diluent, pH 7.2).

was based on the LT gene of ETEC that are pathogenic for humans. Under the established conditions, the primer pair generated the PCR products of predicted length of 298 bp with LT-producing *E. coli* strains of human origin, but not with LT-producing *E. coli* of animal origin, *E. coli* strains that do not produce the LT or other bacterial species. Although there have been some data suggesting that some ETEC strains responsible for human infections may be carried in the intestinal tract of livestock, it is unclear whether animal strains of ETEC are pathogenic for humans (Doyle and Padhye, 1989). Therefore, it was advantageous to use primers based on the LT gene of human strains for PCR detection of ETEC in food samples.

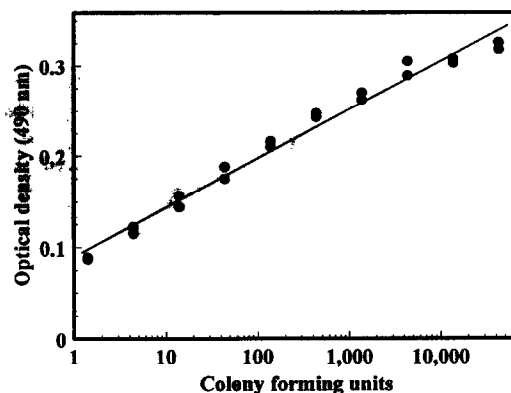


Fig. 2. Relationship between number of colony forming units (CFU) of heat-labile enterotoxin-producing *E. coli* tested by PCR and optical density (OD) of the PCR products at 490 nm in the ELOPH assay. The 2 data points are of 2 replicate samples. The mean OD of the duplicate negative control samples (corresponding to Fig. 1B, lane N) was 0.042 ± 0.001 . The relationship between the number of CFU and OD is described by the line shown, which was obtained from the means of replicate samples. The best-fit line for the data points is described by the equation: $y = 0.196 + 0.000004X$ and has an r value of $+0.593$. The data from this experiment were analyzed by the methods of Wonnacott and Wonnacott, 1990.

Temperature is a critical parameter for primer annealing in PCR (Ausule et al., 1993). In this study, when the primer annealing and extension temperature was lowered from 60 to 55°C, 2 of 4 porcine strains of LT-producing *E. coli* became detectable. Clearly, temperature had an important effect on the degree of the specificity of the PCR. In the study by Wernars et al. (1991), the two 29-mer PCR

Table 2

Detection of heat-labile enterotoxin-producing *E. coli* from experimentally contaminated food samples by PCR and an ELOPH assay^a

Sample type	1.2 × 10 ⁿ CFU added per sample ^b										Negative control ^c	
	8	7	6	5	4	3	2	1	0	−1		
Ground beef	+ ^d	+	+	+	+	+	+	+	− ^e	−	−	−
Chicken	+	+	+	+	+	+	+	+	−	−	−	−
Pork	+	+	+	+	+	+	+	+	+	−	−	−
Raw milk	+	+	+	+	+	+	+	+	+	−	−	−

^a*E. coli*, serotype O25:NM, strain 43886 was used in this experiment.

^bCFU, bacterial colony forming units; each raw milk sample was 25 ml in volume and meat samples had a weight of 25 g; $n = \log$ CFU added from 8 to -1 .

^cNo bacteria were added.

^dPositive signal by visualization on a gel and by a positive ELOPH assay.

^eNo signal in PCR, either by agarose gel or by the ELOPH assay.

primers used for amplifying a 195-bp fragment of the LT gene were based on the sequence of the subunit of the LT gene of porcine origin, and were longer and had higher G-C contents (58.6% and 55.2%, respectively) than the primers used in this study. These investigators used 3 min at 54°C for primer annealing and obtained positive PCR results with all LT-producing strains, including human isolates. Some non-LT-producing *E. coli* cultures also yielded non-specific DNA bands (Wernars et al., 1991). Similarly, Furrer et al. (1990) detected LT-producing ETEC of both human and porcine isolates using 1 min at 55°C for primer annealing in amplifying a 275-bp fragment of the gene coding for the B subunit of the LT gene.

Yamamoto and Yokota (1983) found that the nucleotide sequence of the *toxB* region of the LT gene of human ETEC strain H10407 and that of the *eltB* gene of an LT-producing ETEC of porcine origin are of the same length (375 bp) and differ only in 8 bases. We know of no information about nucleotide sequence homology between human ETEC and strains isolated from other animals besides porcine. The PCR results obtained with the lower annealing and extension temperature in this study shows the possibility that the nucleotide sequences of the 2 porcine ETEC isolates are more homologous with sequences of ETEC of human origin than are the other animal isolates of LT-producing *E. coli* tested.

According to Doyle and Padhye (1989), the ability to produce enterotoxin alone is not sufficient for ETEC to produce illness in humans. The bacteria must express colonization factors that allow the organism to adhere to the small intestine of humans. Because of the differences in the host specificity of colonization factors, it is unlikely that ETEC strains of animal origin will be pathogenic for humans if they do not possess the colonization factors of the type associated with ETEC involved in human illness.

Experiments using dilutions of LT-producing *E. coli*, strain 43886, showed that as few as 1.4 CFU was sufficient to produce a positive signal in both the agarose gel electrophoresis with ethidium bromide staining and the ELOPH assay. The sensitivity of the PCR is similar to that reported by Olive (1989) and Victor et al. (1991). In the study by Wernars et al. (1991), the detection limit was 6 CFU of LT-positive *E. coli* (strain H665/87) in pure culture.

Tests of experimentally inoculated food samples revealed that an initial inoculum of as few as 1.2 to 12 CFU of LT-producing *E. coli* per 25-g (ml) sample gave a positive PCR signal. Although this minimum detectable initial inoculum is similar to that of Wernars et al. (1991), who obtained positive PCR results with an initial inoculum of 3 CFU of the organism in 25-g minced meat, our 8-h culturing procedure would be preferable to the 24-h enrichment used by these researchers. In our study on the growth of inoculated *E. coli* O157:H7 in ground beef and raw milk (Deng and Fratamico, 1995), an 8-h enrichment was found to be optimal for PCR detection of the organism. This enrichment step was, therefore, adapted in this study although there might be differences between the growth kinetics of LT-producing *E. coli* and *E. coli* O157:H7 in the food samples. Certainly, the sensitivity levels obtained with experimentally inoculated food samples may be different from levels obtainable with foods naturally-contaminated with LT-producing ETEC. In our study, freshly grown overnight cultures were used to inoculate the various food

samples. During enrichment, the freshly inoculated bacteria would have growth advantages over the contaminants originally present in the food samples. In naturally-contaminated foods, LT-producing ETEC may be exposed to stressful environments during processing, storage and/or refrigeration. Therefore, the number of total LT-positive organisms after the 8-h enrichment would likely be less than that found with the experimental conditions employed in this study.

It is unclear why the level of detection of ETEC in ground beef/chicken and pork/raw milk samples differed. In our study on *E. coli* O157:H7 (Deng and Fratamico, 1995), each 25-g (ml) of inoculated samples of ground beef and raw milk received 225 ml of modified EC broth with novobiocin (Okrend et al., 1990) and was processed and incubated as described for ETEC-inoculated samples in Section 2. No food effect on the rate of growth of *E. coli* O157:H7 was found. In the present study, the difference in detection levels may be due to a variation in the growth rate of ETEC in different types of food samples, the presence of inhibitory compounds in ground beef and chicken samples which may have had an effect on PCR efficiency, or minor errors in preparing dilutions of the organism used for sample inoculation.

In spite of the numerous advantages of PCR methodology, problems have been encountered when it is applied to food samples (Jones and Bej, 1994). First, Taq DNA polymerase in the PCR assay may be inhibited by substances occurring in food samples. Consequently, extraction and purification of the DNA from the food before PCR have been necessary to remove inhibitory compounds (Jones and Bej, 1994). Second, the level of contamination with pathogenic microorganisms in food samples is always relatively low, particularly when compared to clinical samples. Finally, food samples may contain pathogens that have already been killed during processing or may contain remnants of their DNA, leading to a positive PCR and a false-positive result regarding the presence of a particular pathogen (Wernars et al., 1991). We believe that the combination of enrichment culturing, high-temperature denaturation and PCR used in this study has three advantages. Firstly, the short-term culturing increases the level of viable bacterial cells, thus providing a high sensitivity without undue lengthening of time. Secondly, the high-temperature treatment inactivates some inhibitory substances that might be present in the sample and obviates the need for DNA extraction and purification. Thirdly, the enrichment step dilutes dead cells, thus reducing the risk that the dead cells in the sample may lead to a false-positive PCR product. Other researchers have also found pretreatment of enrichment cultures by extraction and purification of DNA unnecessary before performing PCR for detecting LT-producing *E. coli* from inoculated minced meat (Wernars et al., 1991) and *V. cholerae* from seafoods, fresh fruits and vegetables (Koch et al., 1993). Shirai et al. (1991) successfully used PCR to detect the cholera enterotoxin operon of *Vibrio cholerae* directly from enrichment cultures and stool samples without DNA extraction. In a study by Wang et al. (1992), *Listeria monocytogenes* was detectable by PCR in several foods, such as chicken, but not in soft cheese, confirming that soft cheese contains significant inhibitory substances for PCR. No cheese samples were tested in this study. Further experiments on cheese samples would be worthwhile. In detecting ETEC, it is a

concern that enrichment culturing steps, especially those with selective media might result in loss of genetic information (particularly when located on plasmids) thus giving a false-negative result in the PCR (Candrian et al., 1991; Meyer et al., 1991). The non-selective media LB and BHI seemed to be suitable for growth of LT-producing ETEC strains used in this study. Strains 43886 and 35401, two LT producers, were passaged in LB medium over 30 times in the past 1.5 years in this laboratory and still contain the LT-bearing plasmid. In the experiment with inoculated samples, we did not encounter the loss of the plasmid due to enrichment culturing when using BHI medium.

The ELOPH assay was not only used for confirmation of the identity of the PCR products; the relationship between the number of CFU and the OD of the assay was also examined. The ELOPH assay demonstrated approximately the same sensitivity as agarose gel electrophoresis with ethidium bromide staining. A positive correlation existed between the number of CFU of LT-positive *E. coli* and the OD of the PCR products at 490 nm in the ELOPH assay. A further study will be carried out to determine whether the assay could be used to quantitate the initial and final bacterial load in 'unknown' samples. The ELOPH assay developed in this study is simple, reliable, and does not involve the use of radioactive materials. It is also sensitive enough for routine use. Furthermore, since 96-well microplates are used in the assay, the procedure can be automated (Holodniy et al., 1992). In conclusion, the method described here is sensitive, specific, simple, and rapid. It should therefore be suitable for the routine investigation of food samples for the presence of LT-producing *E. coli*.

Acknowledgements

We thank Geoffrey Letchworth and Zheko Kounev for reviewing the manuscript, Charles Kaspar for providing some bacterial strains, Peggy Greb for preparing photographs, and Dormain Vance for formatting the manuscript. This study was partially supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison and by donations from the food industry.

References

- Ausule, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1993) Current protocols in molecular biology. John Wiley and Sons Inc., New York.
- Candrian, U., Furrer, B., Hofelein, C., Meyer, R., Jermini, M. and Luthy, J. (1991) Detection of *Escherichia coli* and identification of enterotoxigenic strains by primer-directed enzymatic amplification of specific DNA sequences. Int. J. Food Microbiol. 12, 339–352.
- Deng, M.Y. and Fratomico, P.M. (1995) Detection of *Escherichia coli* O157:H7 in meat samples by a multiplex PCR. (in preparation for publication).
- Doyle, M.P. and Padhye, V.V. (1989) *Escherichia coli*, In: Doyle M.P. (Ed.), Foodborne bacterial pathogens. Marcel Dekker Inc., New York, pp. 235–281.

- Evans, D.G., Silver, R.P., Evans Jr., D.J., Chase, D.G. and Gorbach, S. (1975) Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infect. Immun.* 12, 656–667.
- Furrer, B., Candrian, U. and Luthy, J. (1990) Detection and identification of *E. coli* producing heat-labile enterotoxin type 1 by enzymatic amplification of a specific DNA fragment. *Lett. Appl. Microbiol.* 19, 31–34.
- Gannon, V.P.J., King, R.K., Kim, J.Y. and Thomas, E.G. (1992) Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. *Appl. Environ. Microbiol.* 58, 3809–3815.
- Holodniy, M., Winters, M.A. and Merigan, T.C. (1992) Detection and quantitation of gene amplification products by a non-isotopic automated system. *BioTechniques* 12, 37–39.
- Jablonski, E., Moomaw, E.W., Tullis, R.H. and Ruth, J.L. (1986) Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probe. *Nucl. Acids Res.* 14, 6115–6128.
- Jones, D.D. and Bej, A.K. (1994) Detection of foodborne microbial pathogens using polymerase chain reaction methods. In: Griffin H.G. and Griffin A.M. (Eds.), *PCR Technology: Current Innovations*, CRC Press Inc., Boca Raton, FL, pp. 341–365.
- Koch, W.H., Payne, W.L., Wentz, B.A. and Cebula, T.A. (1993) Rapid polymerase chain reaction method for detection of *Vibrio cholerae* in foods. *Appl. Environ. Microbiol.* 59, 556–560.
- Lampel, K.A., Jagow, J.A., Trucksess, M. and Hill, W.E. (1990) Polymerase chain reaction for detection of invasive *Shigella flexneri* in food. *Appl. Environ. Microbiol.* 56, 1536–1540.
- Leong, J., Vinal, A.C. and Dallas, W.S. (1985) Nucleotide sequence comparison between heat-labile toxin B-subunit cistrons from *Escherichia coli* of human and porcine origin. *Infect. Immun.* 48, 73–77.
- Levine, M.M. (1987) *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* 155, 377–389.
- Mahon, J., Murphy, C.K., Jones, P.W. and Barrow, P.A. (1994) Comparison of multiplex PCR and standard bacteriological methods of detecting *Salmonella* on chicken skin. *Lett. Appl. Microbiol.* 19, 169–172.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Meyer, R., Luthy, J. and Candrian, U. (1991) Direct detection by polymerase chain reaction (PCR) of *Escherichia coli* in water and soft cheese and identification of enterotoxigenic strains. *Lett. Appl. Microbiol.* 13, 268–271.
- Neill, M.A., Tarr, P.I., Taylor, D.N. and Trofa, A.F. (1994) *Escherichia coli*. In: Hui Y.H., Gorham J.R., Murrel K.D. and Cliver D.O. (Eds.), *Foodborne Disease Handbook*, Vol. 1. Marcel Dekker, New York, pp. 169–213.
- Okrend, A.J., Rose, B.E. and Lattuada, C.P. (1990) Use of 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide in MacConkey sorbitol agar to aid in the isolation of *Escherichia coli* O157:H7 from ground beef. *J. Food Prot.* 11, 941–943.
- Olive, D.M. (1989) Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. *J. Clin. Microbiol.* 27, 261–265.
- Sack, D.A. and Sack, R.B. (1975) Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. *Infect. Immun.* 11, 334–336.
- Sack, R.B. (1975) Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu. Rev. Microbiol.* 29, 333–353.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, J., Higuchi, R., Horn, G.T., Mullis, K.B. and Ehrlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491.
- Shirai, H., Nishibuchi, M., Ramamurthy, T., Bhattacharya, S.K., Pal, S.C. and Takeda, Y. (1991) Polymerase chain reaction for detection of the cholera enterotoxin operon of *Vibrio cholerae*. *J. Clin. Microbiol.* 29, 2517–2521.
- Swanson, K.M., Busta, F.F., Peterson, E.H. and Johnson, M.G. (1992) Colony count methods. In: Vanderzant C. and Splitstoeser D.F. (Eds.), *Compendium of Methods for the Microbiological Examination of Foods*, 3rd edn., American Public Health Association, Washington, DC, pp. 75–95.

- Victor, T., Du Toit, R., van Zyl, J., Bester, A.J. and van Helden, P.D. (1991) Improved method for the routine identification of toxigenic *Escherichia coli* by DNA amplification of a conserved region of the heat-labile toxin A subunit. J. Clin. Microbiol. 29, 158–161.
- Wang, R.F., Cao, W.W. and Johnson, M.G. (1992) 16 rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. Appl. Environ. Microbiol. 58, 2827–2831.
- Wernars, K., Delfgou, E., Soentoro, P.S. and Notermans, S. (1991) Successful approach for detection of low numbers of enterotoxigenic *Escherichia coli* in minced meat by using the polymerase chain reaction. Appl. Environ. Microbiol. 57, 1914–1919.
- Wonnacott, T.H. and Wonnacott, R.J. (1990) Introductory Statistics, 5th edn. John Wiley and Sons, New York.
- Yamamoto, T. and Yokota, T. (1983) Sequence of heat-labile enterotoxin of *Escherichia coli* pathogenic for humans. J. Bacteriol. 155, 728–733.
- Volken, R.H., Greenberg, H.B., Merson, M.H., Sack, R.B. and Kapikian, A.Z. (1977) Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. J. Clin. Microbiol. 6, 436–444.